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Elevated extracellular calcium levels induce smooth muscle cell matrix mineralization in vitro[1]

HSUEH YANG, GABRIELLE CURINGA, and CECILIA M. GIACHELLI

Department of Bioengineering, University of Washington, Seattle, Washington; and Department of Pathology, University of Washington, Seattle Washington

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Background. Hyperphosphatemia, elevated calcium × phosphorus product (Ca × P), and calcium burden, major causes of vascular calcification, are correlated with increased cardiovascular morbidity and mortality in dialysis patients.

Methods. To address the underlying mechanisms responsible for these findings, we have utilized an in vitro human smooth muscle cell (HSMC) model of vascular calcification. Previous studies using this system demonstrated enhanced calcification of HSMC cultures treated with phosphorus levels in the hyperphosphatemic range, and implicated a sodium-dependent phosphate cotransport-dependent mechanism in this effect. In the present study, we examine the effect of increasing calcium concentrations on HSMC calcification in vitro.

Results. Increasing calcium to levels observed in hypercalcemic individuals increased mineralization of HSMC cultures under normal phosphorus conditions. Importantly, at these total calcium concentrations, ionized calcium levels increased from 1.2 mmol/L to 1.7 mmol/L, consistent with levels observed physiologically in normocalcemic and hypercalcemic individuals, respectively. Furthermore, increasing both calcium and phosphorus levels led to accelerated and increased mineralization in the cultures. Calcium-induced mineralization was dependent on the function of a sodium-dependent phosphate cotransporter, since it was inhibited by phosphonoformic acid (PFA). While elevated calcium did not affect short-term phosphorus transport kinetics, long-term elevated calcium treatment of HSMCs induced expression of the sodium-dependent phosphate cotransporter, Pit-1.

Conclusion. These studies suggest that elevated calcium may stimulate HSMC mineralization by elevating Ca x P product and enhancing the sodium-dependent phosphate cotransporter-dependent mineralization pathway previously observed in HSMCs.

[1]**See Editorial by Towler, p. 2467.**

Key words: hypercalcemia, uremia, chronic kidney disease, vascular calcification, hyperphosphatemia, cardiovascular mortality.

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Over the last few years, a number of studies have focused on the clinical significance of vascular calcification in chronic kidney disease (CKD). Nearly half the deaths in dialysis patients are due to cardiovascular disease [1]. In CKD patients, vascular calcification is significantly associated with coronary ischemic disease [2]. In addition, increased vascular calcification in CKD patients leads to increased arterial wall stiffness and increased pulse pressure both correlating with enhanced cardiovascular mortality in CKD patients [3, 4, 5]. Furthermore, cardiac valve and myocardial calcification are also increased and probably contribute to morbidity and mortality in dialysis patients [6]. Finally, severe medial calcification of small arterioles leads to calciphylaxis, a rare but often fatal syndrome of ischemic necrosis of the skin and adjacent tissues seen almost exclusively in uremic patients [7]. Thus, understanding regulation and potentially controlling vascular calcification in CKD patients is a high priority.

Vascular calcification is currently recognized as a cell regulated process caused by loss of calcification inhibitors and involving osteoblast/chondroblast-like changes in vascular cell gene expression patterns and matrix development (see review in [8]). Stimuli inducing these changes as well as mechanisms for induction are currently under intense investigation. In CKD patients, hyperphosphatemia and elevated calcium x phosphorus (Ca × P) are prevalent and correlate with vascular calcification [2]. Hyperphosphatemia as well as elevated Ca × P product have been linked to the increased cardiovascular mortality risk observed in dialysis patients in a growing number of studies [2, 9, 10]. In addition, serum calcium as well as total calcium load (defined as daily amount of elemental calcium ingested) is often elevated in patients taking calcium-containing phosphorus binders. Hypercalcemia is highly correlated with vascular calcification in people as well as experimental animals [11]. Importantly, several recent studies have shown that increased calcium load (even in the absence of hypercalcemia) is also highly associated with vascular calcification in end-stage renal disease (ESRD) patients. Goodman et al [12] showed that coronary artery calcification was common

and progressive in ESRD patients less than 30 years old, and highly correlated with daily intake of calcium. Likewise, Guerin et al [13] associated arterial and aortic stiffening and vascular calcification with prescribed dose of calcium-based phosphorus binders. Finally, a recent randomized clinical trial comparing a noncalcium containing phosphorus binder (sevelamer) with calcium-based phosphorus binders in a large hemodialysis patient group found that patients receiving sevelamer had median coronary artery and aorta calcification scores unchanged after 1 year as opposed to calcium-treated patients whose coronary arterial calcification scores increased 28% over baseline [14]. Significantly, while both treatments controlled phosphorus levels equivalently, treatment with calcium-containing binders led to an increased frequency of hypercalcemic episodes and greater suppression of serum parathyroid hormone (PTH) levels in hemodialysis patients.

These findings have prompted us to determine whether elevated calcium, phosphorus, or Ca × P directly affect the potential of smooth muscle cells to mineralize their matrices. We previously showed that elevated phosphorus stimulates phosphorus uptake in smooth muscle cells (SMC) via a sodium-dependent phosphate cotransporter resulting in mineralization and phenotypic modulation of vascular SMC (VSMC) cultures to mineralizing cells with osteoblast/chondrocyte-like features [15, 16]. In the present study, we have addressed the effect of increasing calcium in regulating SMC mineralization in vitro.

METHODS

Reagents

Dulbecco's modified Eagle's medium (DMEM) (high glucose, 4.5 g/L of glucose) and fetal bovine serum (FBS) were purchased from Life Technologies, Inc. (Grand Island, NY, USA). Calcium-free media was purchased from Invitrogen (Carlsbad, CA, USA). All chemicals were reagent grade.

Cell culture

Human SMCs (HSMCs) were obtained by enzymatic digestion of fetal and newborn aortas as previously described [17]. Briefly, medial tissues were separated from segments of fetal and newborn aorta obtained at autopsy. Small pieces of tissue (1 to 2 mm^3) were digested overnight in DMEM supplemented with 165 U/mL collagenase type I, 15 U/mL elastase type III, and 0.375 mg/mL soybean trypsin inhibitor at 37°C. Single cell suspensions were placed in 6-well plates and cultured for several weeks in DMEM supplemented with 20% fetal calf serum (FCS) at 37°C in a humidified atmosphere containing 5% CO_2. Cultures that formed colonies were collected at confluence and maintained in growth medium (DMEM containing 15% FBS and 1 mmol/L sodium pyruvate supplemented with 100 U/mL of penicillin and 100 mg/mL of streptomycin; final inorganic phosphorus concentration = 1.4 mmol/L). Purity of cultures was assessed by positive immunostaining for α-smooth muscle actin (α-SMA) and calponin, and absence of von Willebrand factor. Fetal or newborn HSMC cultures were immortalized using the HPV-E6E7 (HFSMCE6E7) as previously described [17] and were used between passages 20 and 30. We have previously determined that immortalization of HSMC does not affect the mineralization capability of these cells, which was comparable to human adult aortic SMC [15].

HSMC were cultured in DMEM containing 2 mmol/L (final concentration) calcium chloride supplemented with 15% FBS and 100 U/mL penicillin and 100 mg/mL streptomycin (growth medium) as described previously [15]. In some experiments, calcium-free DMEM was supplemented with calcium chloride to achieve concentrations stated in the text.

Induction of calcification

HSMC were routinely subcultured in growth medium. At confluence, the cells were switched to the growth medium containing indicated amounts of inorganic phosphorus (adjusted by addition of appropriate amounts of sodium phosphate) and calcium chloride for up to 12 days. The medium was changed every 2 days. For all the calcification experiments, the first day of culture in the calcification medium was defined as day 0.

Quantification of calcium deposition

The calcium content was measured using the Sigma Calcium Kit (Sigma Chemical Co., St. Louis, MO, USA) as previously described [15]. Briefly, cells were decalcified with 0.6 N HCl for 24 hours. The calcium content of HCl supernatants was determined colorimetrically by the *o*-cresolphthalein complexone method (Sigma Chemical Co.). After decalcification, the cells were washed three times with phosphate-buffered saline (PBS) and solubilized with 0.1 N NaOH/0.1% sodium dodecyl sulfate (SDS). The protein content was measured with the BCA Protein Assay Kit (Pierce, Rockford, IL, USA). The calcium content of the cell layer was normalized to total cellular protein.

Determination of spontaneous calcium phosphorus precipitation in culture media

Spontaneous calcium phosphorus precipitation was measured by incubating normal growth media and media with increased Ca x P concentrations in 6-well plates at 37°C for 7 to 10 days in the absence of cells. Media were then collected and centrifuged for 5 minutes at 14,000 × *g*. The supernatant was carefully aspirated and the pellet was dissolved in 0.6 N HCl and measured

with the Sigma Diagnostics colorimetric calcium assay as described above.

Determination of calcium levels

Total calcium in media was determined using atomic absorption performed on Solaar 989 analyzer. Total inorganic phosphorus was measured on the Synchron LX20 Pro Analyzer. Ionized calcium was measured on an AVL 9180 Electrolyte Analyzer. All determinations were made in media prior to incubation with cells. All analyses were performed by the Research Testing Services in the Department of Laboratory Medicine at the University of Washington.

Phosphorus transport assays

Cells were seeded at 10^5cells/well in 24-well plates. At confluence (24 hours after seeding), transport studies were performed at 37°C in Earle's buffered salt solution (EBSS) with 15 mmol/L Hepes adjusted to pH 7.4. The composition of this solution was 143 mmol/L sodium or choline, 5.36 mmol/L potassium, 0.8 mmol/L magnesium, 2 mmol/L calcium, and 125 mmol/L chloride. The cells were washed two times with warm EBSS before the transport assay was started. Transport was initiated by adding 0.5 mL of the above medium containing the labeled substrate $H_3{}^{32}PO_4$ (1 μ Ci/mL) to HSMCs at confluence [15]. After various incubation times, the uptake was stopped by washing the cell monolayer three times with 1 mL of ice-cold stop solution (containing 137 mmol/L NaCl, 14 mmol/L Tris, and 10 mmol/L sodium arsenate). The cells were solubilized with 0.5 mL of 0.1 N NaOH/0.1% SDS, and the radioactivity of 50 uL aliquots was counted by standard liquid scintillation techniques. Protein concentrations were determined with the BCA Protein Assay Kit (Pierce). Finally, the data were normalized to the protein content.

Quantification of mRNA levels

Real-time polymerase chainr reaction (PCR). cDNA was generated by reverse transcription of HSMC mRNA and was amplified using ABI Prism7000 Sequence Detection System according to the manufacturer's suggestion. The Primer Express software (Applied Biosystems, Inc., Foster City, CA, USA) was used to design the PCR primers and the TaqProbe. All the primers were synthesized by Qiagen Inc. (Valencia, CA, USA) and the Taq probes were obtained from Applied Biosystems. All Taq probes had a FAM (fluorochrome reporter) tag at the 5′-end and a MGB (minor groove binder) quencher at the 3′-end. The primers for human type III sodium-dependent phosphate cotransporter (NPC), Pit-1 (Glvr-1) were forward 5′-GGAGGGTGTCAAGTGGT CTG AA-3′ and reverse 5′-ATCTGCCTTATGGAGG ATGAATG-3′. The Pit-1 probe is 5′-CTGATAAAAAT TGTGATGTCTTGG-3′. The primers for human alkaline phosphatase (ALP) were forward 5′-GAACGAGG TCACCTCCATCCT-3′, reverse 5′-TCTCGTGGTGGT CACAATGC-3′. The human ALP probe is 5′-ACGC TGGGAAATC-3′. The primers for the real-time PCR were used at final concentrations of 400 nmol/L and the probes were used at final concentrations of 250 nmol/L. Quantification of gene expression relative to 18S rRNA was calculated using the ΔΔCt method according to the manufacturer's instructions (Applied Biosystems). 18S rRNA was amplified using TaqMan ribosomal RNA control reagents (Applied Biosystems).

Quantitative reverse transcriptase PCR. Human Pit-1 cDNA was amplified using the following primer sequences: forward 5′-TACCATCCTCATCTCGGTGG-3′, reverse 5′-TGACGGCTTGACTGAACTGG-3′.

Northern blotting. Cbfa1 mRNA levels were determined by Northern blotting exactly as previously described [15].

RESULTS

We previously characterized an in vitro HSMC mineralization model that mimics several aspects of vascular calcification observed in vivo, including apatite deposition in and around collagen fibrils, matrix vesicle formation, and agglomeration of apatite crystals to large aggregates [15]. Consistent with previous studies, we found that elevated inorganic phosphorus was a good inducer of HSMC mineralization (Fig. 1A). No apatite accumulated in cultures treated with normal levels of phosphorus (1.4 mmol/L) and calcium (2.0 mmol/L), while increasing phosphorus concentrations to hyperphosphatemic levels (2.6 mmol/L) dramatically increased calcium accumulation in the HSMC cultures.

To determine whether calcium concentration affected HSMC mineralization, we examined matrix calcification in response to treatment with extracellular calcium levels similar to those observed in hypercalcemia. In all cases the final mmol/L calcium concentration was calculated based on the molarity of calcium chloride added. As shown in Figure 1B, treatment of cells cultured in normal phosphorus-containing media (1.4 mmol/L; 4.3 mg/dL) with total calcium concentrations of 2.0 mmol/L (8.0 mg/dL) to 3.0 mmol/L (12.0 mg/dL) led to ionized calcium levels of 1.2 mmol/L (4.8 mg/dL) to 1.7 mmol/L (6.8 mg/dL), respectively, as determined by calcium-sensitive electrode. Importantly, cells treated with increased calcium levels (2.6 and 3.0 mmol/L) and normal phosphorus levels (1.4 mmol/L) deposited increased extracellular mineral, indicating that increasing calcium alone could affect the potential of HSMCs to mineralize (Fig. 1B). Finally, if both calcium and phosphorus were elevated, a dramatic increase in calcification was observed (Fig. 1C).

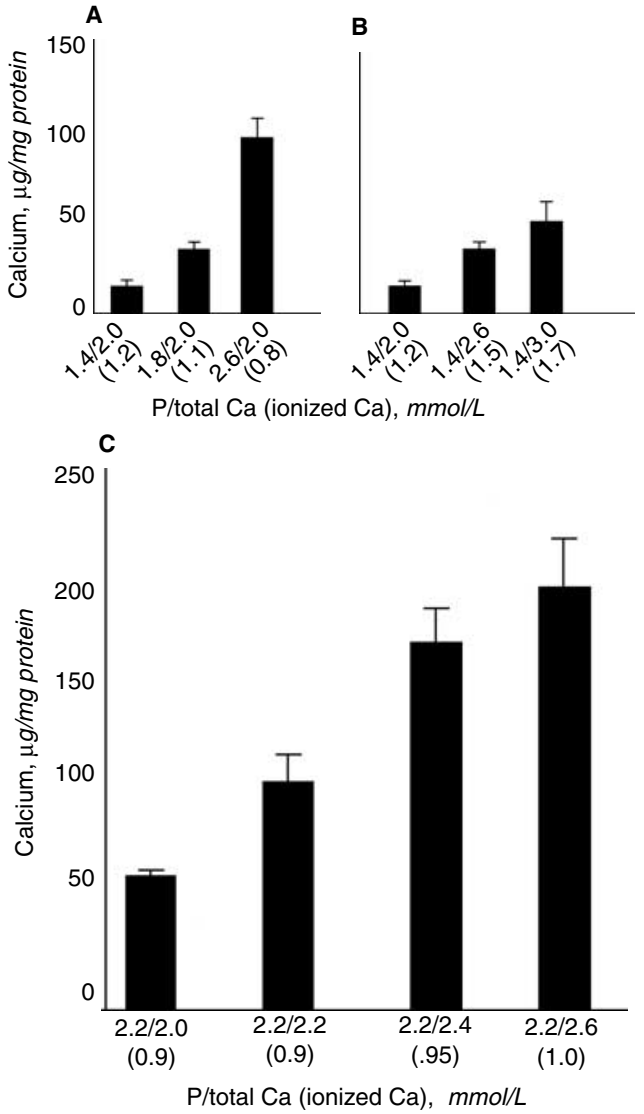

Fig. 1. Calcium (Ca) and phosphorus (P) effects on matrix mineralization of human vascular smooth muscle cells. Human smooth muscle cells (HSMC) were treated with the indicated concentrations of total calcium and phosphorus for 12 days. Extracellular calcium deposition was measured and normalized to corresponding cellular protein. (*A*) Normal calcium and increasing concentrations of phosphorus. (*B*) Normal phosphorus and increasing concentrations of calcium. (*C*) Elevated phosphorus and increasing concentrations of calcium. Values are expressed as a mean ± SD ($N = 3$). Ionized levels of calcium in the culture media were measured as described in the **Methods** section.

In all cases, the final media Ca × P concentrations (prior to incubation with cells) were kept below the solubility product of calcium and phosphorus for calcium phosphorus dibasic or tribasic, such that spontaneous crystallization in the media did not occur. Spontaneous precipitation of calcium phosphorus was assessed as described in the **Methods** section. Levels of calcium and phosphorus at and above 3.2 mmol/L and 2.8 mmol/L, respectively, were not studied because they resulted in spontaneous precipitation in the media.

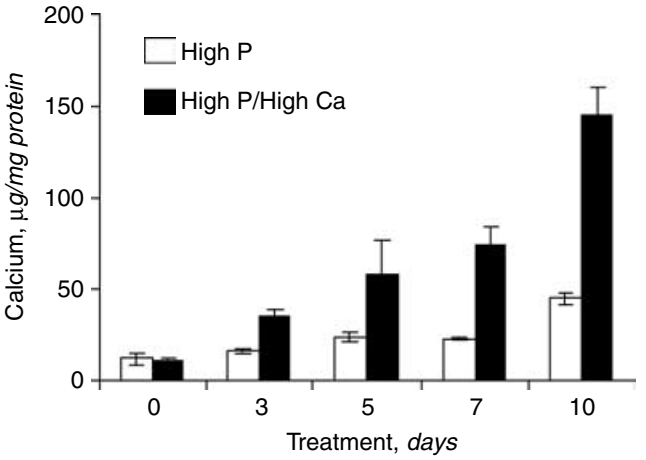

Fig. 2. Time course of smooth muscle cells (SMC) mineralization in the presence of elevated phosphorus (P) with or without elevated calcium (Ca). Human SMC (HSMC) were treated with elevated (2.2 mmol/L) phosphorus and normal calcium (2.0 mmol/L) (□), or high phosphorus and high calcium (2.6 mmol/L) (■), high phosphorus/high calcium. Extracellular matrix calcium deposition was measured at the indicated time points and normalized to corresponding cellular protein. Values are expressed as a mean ± SD ($N = 3$).

To determine whether elevated calcium levels affected the time course of phosphorus-induced mineralization, we cultured cells for the indicated times in 2.2 mmol/L phosphorus and 2.0 mmol/L calcium (high phosphorus) or 2.2 mmol/L phosphorus and 2.6 mmol/L calcium (high phosphorus/high calcium). As shown in Figure 2, a combination of elevated phosphorus and elevated calcium accelerated mineralization in the HSMC. Significant calcification was observed as early as day 3 in cultures treated with high phosphorus and high calcium, compared to day 10 with high phosphorus alone.

To determine the relationship between Ca x P product and HSMC mineralization, data from Figures 1 and 2 were regraphed and a linear regression analysis was performed. As shown in Figure 3, Ca x P product was highly correlated with calcium deposition in HSMC ($R^2 = 0.9811$), however, the relationship was not strictly linear. These findings confirm an important role for Ca x P in HSMC mineralization

To determine whether elevated calcium-induced mineralization was cell-mediated, we performed additional experiments. Since it was previously shown that phosphorus-induced HSMC mineralization was dependent on the activity of a sodium-dependent phosphate cotransporter [15] we tested whether an inhibitor of this system, phosphonoformic acid (PFA), had any effect on calcium-induced mineralization. Interestingly, PFA completely blocked calcium- as well as phosphorus-induced mineralization in HSMC (Fig. 4). This suggested that the observed effects of elevated calcium on HSMC mineralization were cell mediated, required a sodium-dependent phosphate cotransporter and possibly involved regulation of cotransporter function, levels, or both.

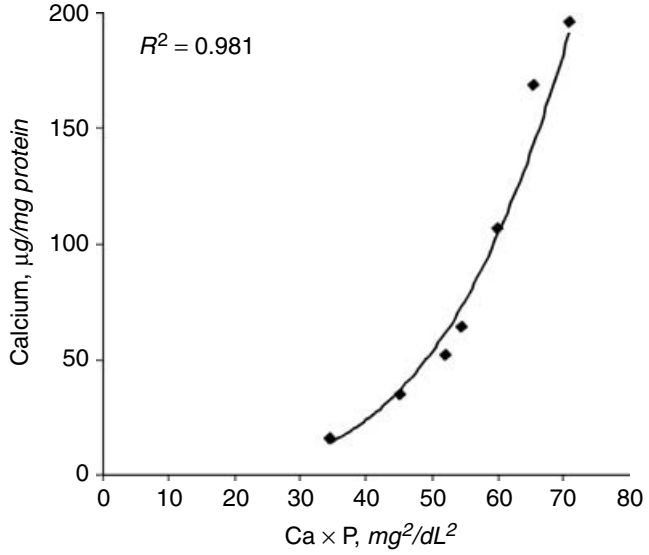

Fig. 3. Correlation between calcium (Ca) deposition into smooth muscle cells (SMC) cultures and Ca × P product. Data from Figures 1 and 2 were regraphed and a regression analysis was performed. Ca × P products (x axis) were calculated from total calcium and *P* values in media prior to incubation with cells. Extracellular matrix calcium deposition as determined from culture lysates is shown on the y axis. A nonlinear, positive relationship between calcium deposition and Ca × P product was noted ($R^2 = 0.9811$).

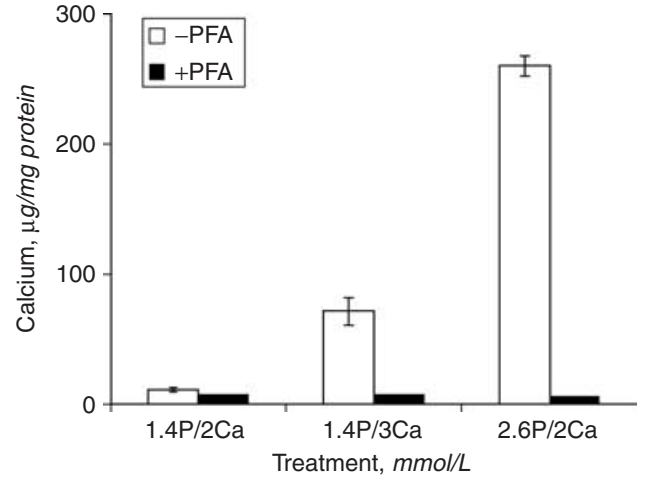

Fig. 4. Effect of phosphonoformic acid (PFA) on elevated calcium (Ca)- and elevated phosphorus (P)-induced smooth muscle cell (SMC) mineralization. Human SMC (HSMC) were treated with the indicated concentrations of calcium and phosphorus in either the presence (■) or absence (□) of 0.5 mmol/L PFA for 10 days. Extracellular matrix calcium deposition was measured and normalized to corresponding cellular protein ($N = 3$). Values are expressed as a mean ± SD.

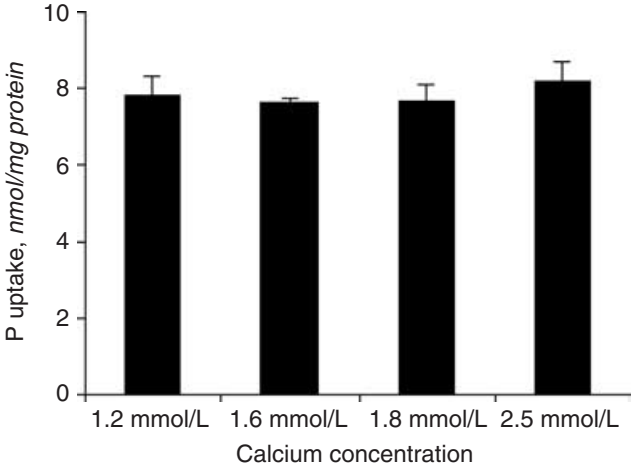

Fig. 5. Effect of extracellular calcium (Ca) levels on phosphorus (P) uptake in human smooth muscle cells (HSMC). HSMC were cultured in the presence of the indicated concentrations of calcium for 24 hours. Phosphorus uptake for 40 minutes was measured as described in the **Methods** section ($N = 3$). Values are expressed as a mean ± SD.

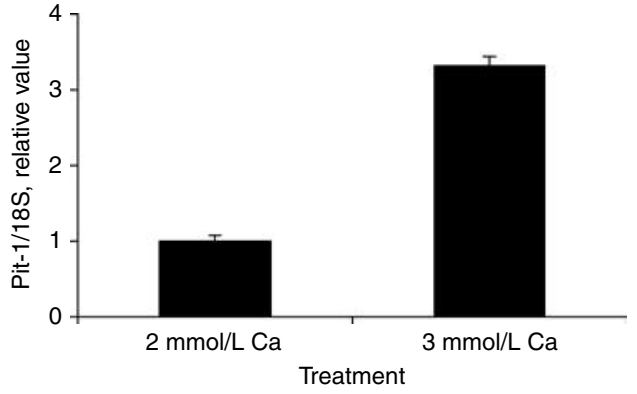

Fig. 6. Expression of Pit-1 in human smooth muscle cells (HSMC). HSMC were cultured with the indicated concentrations of calcium (Ca) for 10 days. Pit-1 transcript levels were measured with real-time polymerase chain reaction (PCR) and normalized to 18S rRNA as described in the **Methods** section. Real-time PCR assays were carried out in triplicate and mRNA values are expressed as a mean ± SD. Results are representative of two separate experiments measuring Pit-1 mRNA levels, by real-time PCR (shown) or quantitative reverse transcriptase PCR (not shown).

To examine these possibilities, phosphorus uptake and transporter mRNA levels were examined in HSMC treated with elevated calcium. As shown in Figure 5, short-term (40 minutes) elevations in extracellular calcium concentration did not alter phosphorus uptake kinetics in HSMCs. This suggested that the transport function of the sodium-dependent phosphate cotransporter was not enhanced by short-term elevation of calcium. Long-term (10 days) treatment of HSMC with elevated calcium-containing medium, however, increased the levels of Pit-1 mRNA, the major sodium-dependent phosphate cotransporter previously shown to be expressed in HSMC [15] (Fig. 6). These studies suggest that increasing calcium enhances HSMC mineralization in a sodium-dependent phosphate cotransporter-dependent manner and up-regulates the type III sodium-dependent phosphate cotransporter, Pit-1.

To determine whether elevated calcium affected the phenotypic state of HSMC, we examined Cbfa1 and alkaline phosphatase mRNA levels at 10 days following treatment with either 3 mmol/L calcium or 2.6 mmol/L

phosphorus. While both mRNAs were detected in elevated calcium-treated cells, levels were not different from those observed in elevated phosphorus -treated cells as determined by real-time PCR (relative mRNA values for alkaline phosphatase 1.02 in elevated calcium-treated cells versus 1.05 in elevated phosphorus-treated cells; relative mRNA values for Cbfa1 1.03 for elevated calcium-treated cells versus 1.2 for elevated phosphorus-treated cells).

DISCUSSION

In the present study, we have determined the effect of elevated extracellular calcium on SMC mineralization in vitro. Increasing calcium to levels observed in hypercalcemic individuals increased mineralization of HSMC cultures under normal phosphorus conditions. Under the conditions used, ionized calcium levels were 1.2 mmol/L to 1.7 mmol/L, consistent with levels observed physiologically in normocalcemic and hypercalcemic individuals, respectively. Furthermore, increasing both calcium and phosphorus levels in the media led to accelerated mineralization as well as a greater extent of mineralization in the cultures compared to elevated phosphorus alone. Most importantly, calcium-induced mineralization were inhibited by PFA, suggesting a cell-mediated, sodium-dependent phosphate cotransporter-dependent mechanism of mineral deposition. While elevated calcium did not affect steady-state uptake of phosphorus in the short-term, long-term treatment of HSMC with elevated calcium increased levels of Pit-1 mRNA, the major sodium-dependent phosphate transporter in HSMC. These data support a model whereby elevations of calcium, in addition to elevating the Ca $\times$ P product, enhance and accelerate HSMC mineralization via increased sodium-dependent phosphate cotransporter levels.

Until recently, vascular calcification was considered to be a passive, degenerative, and end-stage process. However, a number of observations have challenged this paradigm. As in bone formation, matrix vesicles, bone morphogenetic proteins, and noncollagenous bone matrix proteins such as osteopontin, osteonectin, osteocalcin, and matrix Gla protein (MGP) appear in calcified vascular tissues (reviewed in [8]). Likewise, SMCs have the capacity to express bone-related proteins such as Cbfa1, alkaline phosphatase, osteocalcin, and osteopontin and calcify their extracellular matrix under appropriate conditions [15, 16, 18, 19]. Furthermore, a growing number of genes have been associated with vascular calcification based on mouse mutational studies. The MGP-null mouse shows extensive calcification of the arterial tree, indicating that MGP, which is constitutively expressed in arterial SMCs, is normally an important inhibitor of vascular calcification [20]. In addition, the KLOTHO mouse deficient in b-glucosidase [21], the carbonic anhydrase II mutant [22], and osteoprotegerin-null mouse [23] all show enhanced susceptibility to vascular calcification. Finally, structures identical to bone and bone marrow occur frequently in advanced atherosclerotic lesions, calcified cardiac valves, and Monkeberg's sclerosis [24]. These findings suggest that vascular calcification is, in fact, an actively regulated process in which vascular cells normally prevent calcification via elaboration of inhibitory molecules, and under pathologic conditions, can acquire osteoblast/chondrocyte-like properties that can contribute to outright bone formation in vessels.

Likewise, effects of metabolic imbalance on vascular calcification were previously assumed to be mediated entirely through elevation of Ca $\times$ P product and subsequent spontaneous calcium phosphorus precipitation and accumulation in tissues. However, our data suggest that elevated phosphorus and calcium levels are also able to contribute to vascular calcification directly by regulating the susceptibility of VSMC to mineralization. Indeed, we previously showed that phosphorus levels similar to those observed in hyperphosphatemic patients induced SMC to undergo phenotypic transformation to an osteoblast/chondrocyte-like cell that expressed alkaline phosphatase, osteocalcin, and Cbfa1, and mineralized their matrices [15, 16]. Phosphorus-induced phenotypic modulation and mineralization were inhibited by PFA, indicating that a sodium-dependent phosphate cotransporter was involved in the process [15]. These data indicated that cellular phosphorus transport to the extracellular matrix was a key step in nucleating apatite in this model system.

In the present studies, elevated calcium stimulated mineralization of HSMC under normal phosphorus conditions, and accelerated mineralization under elevated phosphorus conditions. Several lines of evidence suggest that the effects of elevated calcium on mineralization are due to both induction of phosphorus uptake as well as elevations in Ca $\times$ P. First, calcium-induced mineralization was inhibited by PFA, similar to phosphorus-induced mineralization, indicating that calcium-induced mineralization was also dependent on the activity of a sodium-dependent phosphate cotransporter. Second, elevated calcium induced expression of the sodium-dependent phosphate cotransporter, Pit-1. Third, mRNA levels of Cbfa1 and alkaline phosphatase in elevated calcium-treated HSMC were similar to those observed in elevated phosphorus-treated HSMC. Finally, elevated Ca $\times$ P strongly correlated with mineralization in HSMC. Thus, we speculate that elevated calcium serves to enhance phosphorus-dependent uptake in HSMC, thereby leading to enhanced apatite nucleation in the extracellular matrix (the rate-limiting step for apatite formation) and HSMC phenotype transition. Once nucleation of apatite occurs, elevated Ca $\times$ P would drive matrix mineralization by accelerating apatite crystal growth via

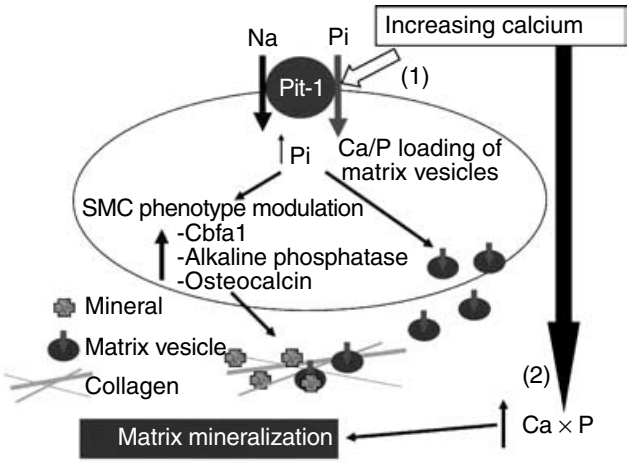

Fig. 7. Hypothetic model for the effects of extracellular calcium (Ca) on vascular smooth muscle cell (VSMC) matrix mineralization. Calcium is proposed to stimulate vascular matrix mineralization in two ways. First, (1) calcium stimulates synthesis of Pit-1, thereby enhancing phosphorus (P) uptake into SMC cell membranes and matrix vesicles. Elevated intracellular phosphorus leads to SMC phenotypic modulation which includes upregulation of osteogenic genes (Cbfa1, osteocalcin, and alkaline phosphatase), and generation of a mineralization-competent extracellular matrix. In addition, increased Pit-1 in matrix vesicles promotes phosphorus loading of matrix vesicles, promoting nucleation of mineral within the extracellular matrix. Second, (2) elevated calcium leads to increased Ca × P product thereby promoting growth of apatite crystals in the matrix via thermodynamic mechanisms.

thermodynamic mechanisms. This proposed model is depicted in Figure 7.

The mechanism whereby elevated calcium increases Pit-1 mRNA levels is not yet clear. Effects of calcium on cells may be mediated through multiple receptors. Of particular relevance to the present studies, Chang et al [25] recently showed that extracellular calcium enhanced terminal differentiation and mineralization of osteoblast precursor cells through a calcium receptor-sensitive mechanism. Likewise, L-type calcium channels have been recently implicated in calcium-mediated regulation of osteoblastic differentiation [26]. Together with our own, these studies suggest that elevated calcium may directly affect signaling pathways that regulate the propensity of HSMC to mineralize their matrices.

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Reprint requests to Cecilia M. Giachelli, Bioengineering Department, Box 351720, Bagley Hall, Room 479, University of Washington, Seattle, WA 98195.
E-mail: ceci@u.washington.edu

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